Nanobodies targeting and modulating CXCR4 and ACKR3

Bobkov, V.

2021

document version
Publisher's PDF, also known as Version of record

CHAPTER 3

Display technologies for generation of Ig single variable domains

Vladimir Bobkov, Bas van der Woning, Raimond Heukers, Hans de Haard, Martine J. Smit

Adapted from:
Bobkov et al., Display technologies for generation of Ig single variable domains. *Methods Mol Biol.* 2018; 1827:129-144

1. ABSTRACT

Variable fragments of heavy-chain only antibodies (VHH) found in Camelids are valuable research tools in pharmacology, biotechnology, and are being developed for the clinic to treat patients with autoimmune and infectious diseases or cancer. Their single-domain nature and biochemical properties greatly facilitates the development process. The most common technology to select single-domain antibody fragments is phage display following active immunization of llamas or other members of *Camelidae* family. Selection of VHH from immune phage libraries is a rapid approach to discover a broad panel of *in vivo* matured antigen-specific clones with comprehensive functionalities. In this chapter, we describe a detailed protocol for construction of VHH immune libraries and phage display selection against antigens in their native conformation.

2. INTRODUCTION

Since the discovery of heavy-chain-only antibodies (HCAbs) in llamas in 1993 by Hamers-Casterman *et al*. [122], the potential of single-domain antibodies, also called Variable Heavy-chain region of a Heavy-chain only antibody or VHH, has been widely explored in the treatment and diagnosis of cancer, infectious and autoimmune diseases, as well as in structural and functional studies of GPCRs [132, 133, 244]. Unlike the conventional antibodies, the HCAbs of Camelids lack CH1 domain and cannot pair with light chains. Therefore, variable fragments of such HCAbs are represented by small, about 15kDa, single-domain VHH, which can be easily expressed recombinantly in prokaryotic systems with fully retained antigen binding properties and excellent stability in solution. Their single-domain nature and favorable biochemical properties make them ideal candidates for an affinity selection via in vitro antibody display.

To raise a specific immune response in llamas, an immunization step can be performed by various methods depending on a target and its available forms. The most straightforward approach is immunization with purified recombinant protein. Nevertheless, many transmembrane targets are dependent on the cellular membrane to maintain a correct native conformation and are generally not available as purified full-length proteins. In this case, immunization with cells or lipo-particles overexpressing the protein of interest can be used as an alternative method. Additionally, DNA immunization approach was successfully applied for generating an immune response against the targets with multiple transmembrane domains such as GPCRs [116, 245]. The advantage of immunization with recombinant protein or DNA over whole cell immunization consists in the lack of non-specific off-target immune response against multiple components of the injected cells, which are also exposed to the immune system of llamas.

After immunization, RNA is isolated from Peripheral Blood Mononuclear Cells (PBMC) and VHH fragments, undergone somatic maturation, can be rescued in a single PCR step. After cloning into a phage vector, single variable domain antibody fragments are fused to the minor coat protein pIII (Figure 1). It was already shown that even screening of individual clones from such a library obtained by immunization with purified antigen yielded a high percentage of target specific clones varying between 6 and 20%, even without prior enrichment with biopanning [246]. However, when produced on phages, multiple copies of VHH per a phage particle can be found resulting in avid binding of the phage to a coated target during the phage selection step. Direct coating of a recombinant protein results in the immobilization of many more copies of target molecules as compared to the coating of cells or lipoparticles, where only limited number of target molecules are present, making the avid phage binding a major advantage during the selection step. High density coating of recombinant proteins in combination with multiple display of single variable domains often results in the selection of clones with suboptimal binding affinity. To prevent this, an antigen concentration can be controlled by the phage selection in solution with biotinylated target (most preferably monomeric) followed by capturing phage-target complexes with streptavidin pre-coated on 96-well plates or magnetic beads.

In this chapter, we provide a detailed protocol for DNA immunization of llamas followed by construction of VHH immune libraries combined with subsequent phage display technology based

on M13 filamentous phage as a robust and the most commonly used technique for VHH selection. Additionally, a protocol for phage selection on immobilized VLPs derived from cells overexpressing a GPCR of interest is also described, providing a great insight into the potential of VHH fragments even against complex targets.

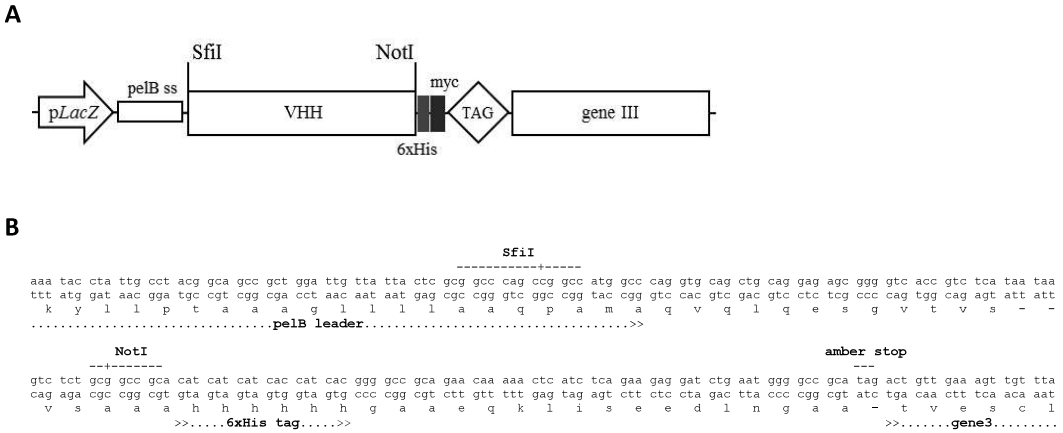

Figure 1. Phagemid vector for VHH phage display. *Schematic representation (A) and nucleotide sequence (B) of VHH cloning site. The cloning site consists of signal sequence (pelB ss), hexahistidine tag (6xHis), and cMyc tag (myc). VHH genes can be cloned via SfiI and NotI restriction sites as fusions with gene III to be displayed on the surface of phages. Amber stop codon (TAG) between VHH and bacteriophage gene III allows the production of soluble VHHs fused with 6xHis and cMyc tags in a non-suppressor strains of E. coli. The expression cassette is under control of the LacZ promotor (pLacZ).*

3. MATERIALS

Prepare all solutions using ultrapure distilled deionized water (ddH_2O). Autoclaving were performed at 121°C, 15 lb/in.^{2}, 20 min. Prepare and store all reagents at room temperature (unless indicated otherwise).

3.1 DNA Immunization of llamas

1. Mammalian expression vector encoding the target of interest such as pcDNA3.1 (ThermoScientific) or similar.
2. EndoFree Plasmid Giga Kit for plasmid DNA purification (Qiagen).
3. Sterile endotoxin-free water.
4. 1-ml syringes for injections.
5. Anesthetic agents: Xylazine and Ketamine.
6. System for *in vivo* intradermal electroporation Agile Pulse ID (Harvard Apparatus).
7. Needle array for electroporation: 6 needles with 4 mm gap and 2 mm length (Harvard Apparatus).
8. Anticoagulants such as EDTA, Heparin, Citrate.
9. Centrifuge.

3.2 RNA Extraction from Peripheral Blood Mononuclear Cells

1. 200 ml of blood from immunized animals.
2. Dulbecco's Phosphate Buffered Saline without Ca^{2+} and Mg^{2+} (Sigma-Aldrich).
3. Pancoll separating solutions (PAN-Biotech).
4. RNeasy maxi kit (Qiagen).
5. RNase-free DNase set (Qiagen).
6. Needle and syringe for cell lysate homogenization.
7. 96% ethanol, HPLC grade.
8. 2 M Sodium Acetate, pH 4.
9. MilliQ H_2O.
10. 50 ml tubes.
11. Centrifuge.

3.3 cDNA Preparation by RT-PCR

1. SuperScript III First-Strand Synthesis System for RT-PCR, containing: Random hexamers, 10X RT buffer, Mg2Cl, DTT, dNTP mix, SuperScript Reverse Transcriptase, and RNaseOUT (Invitrogen).
2. RNase H (Applied Biosystems).
3. UV-Vis spectrophotometer
4. Centrifuge.
5. PCR thermocycler.
6. MilliQ H_2O.
7. RNase free tubes, pipette tips, and flow hood.

3.4 VHH Amplification via PCR

1. 100 µM Hinge-07 primer: TGCGGCCGCGGAGCTGGGGTCTTCGCTGTGGTGCG (NotI recognition site is underlined).
2. 100 µM Hinge-08 primer: TGCGGCCGCTGGTTGTGGTTTTGGTGTCTTGGGTT (NotI recognition site is underlined).
3. 100 µM 5518 primer: CTCGCAACTGCGGCCCAGCCGGCCATGGCCSAGGTGCAGSTGCAGGAGTCGGG (SfiI recognition site is underlined; *see* Note 1).
4. 100 µM 5519 primer: CTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCGGCAGGAGTCGGG (SfiI recognition site is underlined).
5. 100 µM 5520 primer: CTCGCAACTGCGGCCCAGCCGGCCATGGCCSAGGTGCAGCTSGTGGAGTCTGG (SfiI recognition site is underlined; *see* Note 1).
6. 100 µM 5521 primer: CTCGCAACTGCGGCCCAGCCGGCCATGGCCSAGTTGCAGSTGGTGGAGTCTGG (SfiI recognition site is underlined; *see* Note 1).
7. 100 µM 5522 primer: CTCGCAACTGCGGCCCAGCCGGCCATGGCCSAGTTGCAGCTGGTGGAGTCTGG (SfiI recognition site is underlined; *see* Note 1).
8. Phusion High-Fidelity DNA Polymerase kit, containing: Phusion HF Buffer, dNTPs, and Phusion Polymerase (ThermoScientific).

9. Nucleospin Extract II purification kit (Macherey Nagel).
10. MilliQ H_2O.
11. System for agarose electrophoresis.
12. Centrifuge.
13. PCR thermocycler.

3.5 Enzymatic Digestion of VHH Sequences and Ligation into Phagemid Vector

1. NotI and SfiI restriction enzymes and corresponding buffers (ThermoScientific).
2. T4 DNA ligase and corresponding buffer (ThermoScientific).
3. Phagemid vector for phage display with an appropriate cloning sites (Figure1).
4. Nucleospin Extract II purification kit (Macherey Nagel).
5. MilliQ H_2O.

3.6 Transformation of TG1 Cells and Production of Phages

1. Electroporation Apparatus MicroPulser with 0.1 cm Gene Pulser Cuvette (Bio-Rad) or any other suitable system.
2. TG1 electrocompetent *E.coli* (Lucigen).
3. Recovery Medium (Lucigen).
4. 2-YT medium: 16 g Tryptone, 10 g Bacto Yeast Extract, 5 g NaCl, add MilliQ H_2O to 1L, adjust pH to 7.0.
5. 2-YT medium supplemented with 2% glucose and 100 µg/ml ampicillin.
6. 2-YT medium supplemented 100 µg/ml with ampicillin and 25µg/ml kanamycin.
7. VCSM 13 helper phage, 10^{13} CFU/ml (Agilent Technologies).
8. Phosphate Buffered Saline (PBS): 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, add MilliQ H_2O to 1L and adjust pH to 7.4.
9. 20% PEG solution in 2.5 M NaCl.
10. 60% glycerol solution.
11. Ice.
12. Shaking incubator.

3.7 VHH Selection by Biopanning Technique on Virus-Like Particles

1. 96-well MaxiSorp flat-bottom plates and sealers for 96-well plates (ThermoScientific).
2. 96-well low binding V-bottom Assay Plates (Costar).
3. Target and null VLPs from the same cell background (Integral Molecular).
4. PBS (see Subheading 2.6, item 7).
5. PBS with Tween-20 (PBS-T): PBS solution with 0.05% Tween-20 (*see* Note 17).
6. Blocking Solution: PBS solution with 1% dry skimmed milk.
7. Trypsin solution: PBS solution with 1% w/v Trypsin (Sigma). Aliquots can be stored at-20°C.
8. Trypsin inhibitor 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) solution: ddH_20 with 4 mg/ml AEBSF (Sigma). Aliquots can be stored at-20°C.

9. 2-YT medium (*see* Subheading 2.6, item 4).
10. 2-YT medium supplemented with 2% glucose and 100 µg/ml ampicillin.
11. TG1 *E.coli* (Lucigen).
12. LB-agar plates: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L Agar.
13. Shaking platform.
14. 50-ml tubes, filtered tips, pipettes.

4. METHODS

4.1 DNA immunization of llamas

The cDNA encoding the target of interest must be cloned into a mammalian expression vector, e.g. pcDNA3.1, under the control of a strong promoter. The plasmid DNA for immunization must be reconstituted in sterile endotoxin-free water and stored at-20°C. Llamas are housed with water and food *ad libitum* and immunized 6 times in total with two-week intervals. Blood sampling must be performed with anticoagulants: EDTA, Heparin or Citrate. All animal studies must be conducted in accordance with national legislative regulations and after a local ethical approval.

1. Purify the plasmid DNA using EndoFree Plasmid Giga Kit following the manufacturer's instructions.
2. Reconstitute the plasmid DNA into endotoxin-free water at the concentration of 2 mg/ml, aliquot and store at-20°C (see Note 2).
3. Before the first immunization, collect 10 ml of blood from animals using anticoagulants to prevent blood clotting. Centrifuge the blood sample at 1000 x g, aspirate blood plasma and store at-80°C as pre-immune plasma sample for further evaluation of specific immune response (see Note 3).
4. Before each immunization step, llamas are anesthetized for approximately 30 min with an intramuscular injection of 1.5 ml Hella-Brunner mix with 500 mg Xylazine and 150 mg Ketamine.
5. Inject 1 ml of plasmid DNA (2 mg/ml) intradermally, divided over at least 8-10 injection spots.
6. Right after the DNA injection, an electric pulse of 450 V with a resistance below 3000 Ω are given using the Agile Pulse In Vivo electroporation system and parallel-needle array electrodes.
7. After two weeks repeat from step 4.
8. Four days after the last immunization round (*see* **Note 3**), collect 200 ml of blood and proceed to PBMC isolation.

4.2 RNA Extraction from Peripheral Blood Mononuclear Cells

For PBMC separation use the blood from immunized animals with anticoagulants and diluted with a physiological saline solution (PBS without Ca^{2+} and Mg^{2+}).

1. Separate PBMC with Pancoll solution according to the instructions provided by the manufacturer.
2. Extract RNA from the PBMC with RNeasy maxi kit following the manufacturers' instructions (*see* Note 4).

3. Store the RNA at-80°C precipitated with 2.5 volume of 96% ethanol and 0.1 volume of 2 M Sodium Acetate, pH 4.

4.3 cDNA Preparation by RT-PCR

For the RT-PCR reaction use SuperScript III First-Strand Synthesis System. RNA must be dissolved in water and be RNase free to avoid degradation during RT-PCR step. Use RNase free materials and equipment.

1. Approximately 80 µg of RNA needed for construction of one library to completely cover the VHH diversity. Take the needed amount of RNA from the step 3.2 and centrifuge at max speed in a table top centrifuge for 10 min to sediment the RNA from ethanol and Sodium Acetate solution.
2. Wash the pellet with 70% cold ethanol and centrifuge again for 10 min.
3. Air-dry the pellet for 10 min at room temperature and resuspend in ddH_2O (*see* Note 5).
4. Measure RNA concentration by UV spectroscopy at $OD_{260\ nm}$ (*see* Note 6).
5. To denature nucleic acids and allow hexamers to anneal, set up a mix of RNA, hexamers, and dNTPs in a total volume of 180 µl: 18 µl Random hexamers (50 µM), 18 µl dNTPs (10 mM), 80 µg RNA, and ddH_2O to the final volume. Aliquot 20 µl of the mix per PCR tube and heat the tubes in a thermocycler for 5 min at 65°C and then for 1 min at 1°C.
6. For cDNA synthesis, prepare RT-PCR mix in a total volume of 180 µl: 36 µl RT buffer (10x), 72 µl Mg_2Cl (25 mM), 36 µl DTT (100 mM), 18 µl RNaseOUT (40 U/µl), and 18 µl SuperScript III Reverse Transcriptase (200 U/µl). Add 20 µl of the mix to each of the PCR tubes from the previous step. Incubate the tubes in a thermocycler as follows: 50 min at 50°C, 5 min at 85°C, and keep on pause at 1°C.
7. To remove residual RNA, pool the samples from the RT-PCR reaction together, add 3.6 µl RNase H (10 U/µl), and incubate for 20 min at 37°C.
8. Store the cDNA at-20°C.

4.4 VHH Amplification via PCR

During this step a repertoire of VHH sequences are amplified in 6 different combinations with the specific primers encoding the beginning of framework region 1 (named as 5518, 5519, 5520, 5521, 5522) and the primers to two different hinge regions of llama IgG2 and IgG3 (Hinge-07 and Hinge-08) [247]. For each set of primers 2 reactions are performed (12 reactions in total). The primers contain SfiI and NotI restriction sites for subsequent cloning into a phagemid vector. For the PCR reaction use Phusion High-Fidelity DNA polymerase.

1. Set up a PCR mix for 12 reactions in a final volume of 50 µl: 120 µl Phusion HF buffer (5x), 60 µl dNTPs (2mM), 240 µl ddH_20, 48 µl cDNA, and 12 µl Phusion polymerase (2 U/µl). Aliquot 40 µl to 12 PCR tubes and add VHH specific primers, 2 reactions for each set of primers, as follows: (a) 5 µl primer Hinge-07 (5 µM), 2.5 µl primer 5518 (5 µM), and 2.5 µl primer 5519 (5 µM); (b) 5 µl primer Hinge-07 (5 µM), 2.5 µl primer 5520 (5 µM), and 2.5 µl primer 5522 (5 µM); (c) 5 µl primer Hinge-07 (5 µM), 2.5 µl primer 5521 (5 µM), and 2.5 µl primer 5522 (5 µM); (d) 5 µl primer Hinge-08 (5 µM),

2.5 µl primer 5518 (5 µM), and 2.5 µl primer 5519 (5 µM); (e) 5 µl primer Hinge-08 (5 µM), 2.5 µl primer 5520 (5 µM), and 2.5 µl primer 5522 (5 µM); (f) 5 µl primer Hinge-08 (5 µM), 2.5 µl primer 5521 (5 µM), and 2.5 µl primer 5522 (5 µM).

2. Set the following PCR conditions: 3 min at 98°C; maximum 30 cycles of 30 sec at 98°C, 30 sec at 60°C, 55 sec at 72°C; 10 min at 72°C; cooling at 4°C.
3. Run the PCR products on 1.2% agarose gel. Cut out of the gel the bands corresponding to the size of amplified VHHs (400-450 bp). Pool all 12 PCR products and purify with a gel extraction kit.
4. Store the purified PCR products at-20°C.

4.5 Enzymatic Digestion of VHH Sequences and Ligation into Phagemid Vector

The PCR products and phagemid vector (Figure 1) need to be digested with SfiI and NotI restriction enzymes to allow subsequent direct cloning into the vector with cohesive ends.

1. Digest all the PCR products from the step 3.4 with 5 µl NotI (10 U/µl) with an appropriate buffer in the final volume of at least 50 µl for 4 h at 37°C.
2. Purify the reaction using a standard kit.
3. Digest the product of NotI restriction with 5 µl SfiI (10 U/µl) with an appropriate buffer in the final volume of at least 50 µl for 3 h at 50°C.
4. Purify the reaction using a standard kit.
5. Measure the concentration of the digested DNA with the absorbance of 260 nm.
6. Set up a ligation reaction in a final volume of 200 µl: 2 µg digested vector, 0.5 µg digested insert, 20 µl T4 DNA Ligase Buffer (10x), 5 µl T4 DNA ligase (5 Weiss U/µl), and ddH_2O to the final volume. Incubate the ligation mix at RT for 3 h or at 16°C overnight.
7. Perform an additional ligation step by adding: 5 µl T4 DNA Ligase Buffer (10x), 2.5 µl T4 DNA ligase (5 Weiss U/µl), and 42.5 µl ddH_2O. Incubate at 37°C for 2 h.
8. Purify the reaction using a standard kit and elute with 28 µl ddH_2O for subsequent electroporation of bacterial cells.
9. Store the ligation product at-20°C if needed.

4.6 Transformation of TG1 Cells and Production of Phages

The following step is performed with MicroPulser from Bio-Rad in 0.1 cm cuvettes with pre-programmed EC1 settings for *E. coli* (1.8 kV, 1 pulse). Any other electroporation system suitable for *E. coli* can be applied following the manufacturer's recommendations.

1. Put electrocompetent TG1 cells, the purified ligation product, and electroporation cuvettes on ice for 15 min.
2. Set the MicroPulser to EC1 program.
3. Add all the ligation product (28 µl) to 100 µl of the electrocompetent cells. Avoid extensive pipetting. Transfer the mix to 3 precooled electroporation cuvettes (42 µl per cuvette) and tap the suspension to the bottom.
4. Place a cuvette in the chamber and transform the cells.
5. Immediately add 1 ml of Recovery Medium and gently resuspend the cells. Transfer the suspension to a 50-ml tube (*see* Note 7). Repeat for the rest cuvettes.
6. Rinse all 3 cuvettes one more time with 1 ml of Recovery Medium to collect the rest of the cells.
7. Recover the electroporated bacteria by incubating the cells at 37°C for 30 min while shaking (*see* Note 8).
8. Transfer the recovered cells to 300 ml of 2-YT medium with 2% glucose and ampicillin. Incubate at 37°C with shaking (*see* Note 9).
9. For direct production of phages when $OD_{600\,nm}$ reaches 0.5-0.6 (*see* Note 10), transfer 100 ml of the culture into a new flask and add 20 µl of VCSM13 helper phage (*see* Note 11).
10. Incubate at 37°C without shaking to infect the cells with phages. Then incubate another 30 min with shaking.
11. Centrifuge the cell suspension for 10 min at maximum speed and resuspend the pellet in 400 ml 2-TY with ampicillin and kanamycin (without glucose).
12. Incubate at 28°C overnight to produce phages displaying VHHs.
13. On the next day, collect 50 ml of the night culture and centrifuge at 4800 x g for 15 min at 4°C.
14. To precipitate phages collect 40 ml of the supernatant from the previous step, add 10 ml of 20% PEG solution and incubate on ice for 30 min.
15. Centrifuge at 4800 x g for 15 min, discard the supernatants and resuspend precipitated phages in 1 ml PBS.
16. Spin down in a table-top centrifuge at max speed for 3 min to get rid of bacterial cell debris (*see* Note 12).
17. Collect 1 ml of the supernatant with phages for glycerol stocks or store at 4°C for a subsequent selection round.

4.7 VHH Selection by Biopanning Technique on Virus-Like Particles

Virus-like particles (VLPs) are non-infectious particles containing viral protein core surrounded by lipid bilayer with the transmembrane protein of interest in its native conformation. VLPs are widely used in phage and yeast display panning procedures as an alternative to live cells, membrane fractions, and purified transmembrane proteins solubilized in detergent. The concentration of transmembrane target protein on the surface of VLPs is highly enriched comparing to live cells making them a perfect tool for the selection and screening of antibodies against complex transmembrane targets such as GPCRs [116, 214]. There are several companies providing commercially available VLPs. In the protocol bellow, we use VLPs provided by Integral Molecular.

In Figure 2 we demonstrate an example of the results after two rounds of VHH selection from a llama naïve library where a 1000-fold enrichment on CXCR4-positive over CXCR4-negative (null) VLPs is shown. Enrichment is expressed as the fold-difference in phage titer of the phage output after selection on the target over selection on the control. Though the example is taken from a selection with a naïve library, the one should consider a similar positive outcome after 1-2 panning rounds with an immune library. The figure was kindly provided by Fair Journey Biologics.

All the incubation steps in a 96-well MaxiSorp plate for selection are carried at room temperature with shaking at 450-600 rpm and sealed with an adhesive tape to avoid evaporation unless otherwise specified. Perform all the work with phages in a laminar flow hood to avoid contamination of the lab. Use only filtered tips for pipetting. Discard all the solutions containing phages into a beaker with Umonium or bleach.

1. Coat MaxiSorp plate with 2 and 20 Units/well of target and null VLPs in PBS incubating overnight at 4°C without shaking (*see* Note 13).
2. Prepare a fresh overnight culture of TG1 cells from a single colony grown on LB-agar plate. Grow the culture in 50 ml 2-TY overnight at 37°C (*see* Note 14).
3. Next day, wash the MaxiSorp plate twice with PBS.
4. Fill in the wells with 200 µl of blocking solution and incubate for 1-2 h.
5. Remove the blocking solution by inverting the plate over a waste beaker and wash the plate twice with PBS.
6. To allow phages to bind to the coated VLPs, add 100 µl of phages in blocking solution to the wells with target and null VLPs and incubate for 2 h (*see* Note 15).
7. In the same time, prepare logarithmically grown TG1 cells by diluting the overnight culture (see step 3) 200-fold in fresh 2-TY and incubate at 37°C with shaking until $OD_{600\ nm}$ reaches 0.5. This culture of TG1 in logarithmic growth phase will be used for rescuing eluted phages and infection with titrations of phages from input (before selection round) and output (after selection round) (*see* Note 16).
8. Remove the phage solution and tap the plate thoroughly on a tissue paper.
9. To remove unbound and low-affinity phages, wash the wells 5 times with 200 µl PBS-T solution. During the last wash incubate the plate for 5 min at room temperature while shaking at 450-600 rpm.

10. Repeat the step 9.
11. Repeat the step 9 but instead of PBS-T, use PBS.
12. To elute bound phages add 150 µl/well of 1% trypsin solution and incubate for 30 min (*see* Note 18). During the elution step, prefill a low-binding 96-well plate with 7.5 µl trypsin inhibitor solution (AEBSF). Transfer the eluted phages to the plate with trypsin inhibitor.
13. Make serial 10-fold dilutions of output (eluted) and input phages with 5 µl phages and 45 µl 2-TY. Titrate output from 10^{-1} to 10^{-6}, and input from 10^{-1} to 10^{-12}. Add 50 µl TG1 in the logarithmic growth phase (see step 8) to the dilution series and incubate at 37°C for 30 min to let the phages infect the cells. Spot 5 µl of each dilution on LB-agar plates with ampicillin and glucose, let the spots dry and incubate the plates upside down at 37°C overnight.
14. Rescue eluted phages from the wells coated with target VLPs for amplifying the selected clones and preparing phages for subsequent rounds of selection. Transfer 75 µl of eluted phages to 1 ml of TG1 in the logarithmic growth phase (see step 8) in 50 ml tube. Incubate for 30 min at 37°C without shaking.
15. Add 10 ml of 2-TY with 2% glucose and ampicillin and incubate overnight at 37°C with shaking.
16. The next day, make glycerol stocks of the overnight cultures, analyze the LB-agar plates with input and output titrations and compare with negative controls (Figure2). In case of no specific enrichment observed, an additional round of selection needs to be performed.

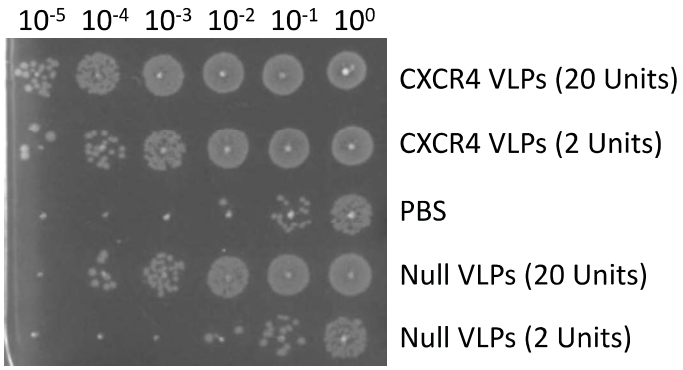

Figure 2. Results of two-round selection from a VHH naive library against a transmembrane target on VLPs (CXCR4, GPCR Class A). *Presented as colonies on a LB-agar plate after titration of phages eluted with trypsin onto E. coli TG1. After overnight incubation specific 103-104 -fold enrichment was observed. Figure provided by Fair Journey Biologics (Porto, Portugal).*

5. NOTES

1. Degenerated oligonucleotide primers are used to cover the natural variability in the VHH sequences. S is for C and G.
2. The amount of DNA needed for 6 immunization rounds is 12 mg per animal. Prepare the plasmid DNA in excess to avoid problems during pipetting.
3. After the third immunization, collect 10 ml of blood, prepare a plasma sample as described in the Subheading 3.1, item 1, and evaluate the specific immune response by measuring the titer against the target of interest via ELISA or FACS. If a significant immune response can be demonstrated in comparison with the pre-immune plasma from the Subheading 3.1, item 1, the number of immunizations can be limited to 4. Otherwise, follow the immunization procedure up to 6 times.
4. RNA integrity could be checked by running an aliquot of 2-4 µl on 1% TAE agarose gel. Three bands of RNA should be observed at 1500 bp, 800 bp, and 200 bp corresponding to 28S, 18S, and 6S rRNA, respectively. Residual DNA might be observed at above 10000 bp. To avoid RNA degradation, gel electrophoresis tanks, trays and combs must be cleaned with 0.1% SDS for at least 1 hour and rinsed with ddH_2O thoroughly.
5. To avoid losing the pellet of RNA, aspirate the supernatants after each centrifugation step and transfer into clean tubes. Dissolving of precipitated RNA in water takes time. Incubate RNA in water for at least 10-15 min at 60°C tapping with fingers every 3-5 min.
6. $OD_{260\ nm}$ value of 1 is equivalent to 40 µg/ml single-stranded RNA. The ratio of $OD_{260\ nm}/OD_{280\ nm}$ is used to verify the purity of RNA. The ratio value of 1.8 – 2.1 indicates pure RNA material.
7. This step is very crucial for recovering transformed *E. coli*. The time between applying a pulse and transferring cells to recovery medium must be as short as possible. Delay in 1 minute might cause a 3-fold drop in transformation efficiency.
8. A library size can be evaluated by taking an aliquot of recovered bacteria, performing a ten-fold serial dilutions and plating on LB-agar dishes with ampicillin and glucose. After overnight incubation at 37°C, the number of colonies formed within a certain dilution can be used to calculate the library size. In our experience with immune libraries it should be around 10^7-10^8 CFU. This is based on the fact that we take a blood sample of around 200 ml for the isolation of the PBMCs. It is expected that around 10^7-10^8 of B-cells are present in such a blood sample. A large amount of RNA, typically 80 µg, is taken for preparation of randomly primed cDNA. Then again, a large amount of the cDNA is used as a template for the PCR of VHH with relatively small number of cycles. Hereby, the VHH diversity sampled from the animals is maintained at every step from cDNA synthesis to PCR amplification and cloning.

The percentage of clones with correct VHH insert can be evaluated via colony PCR with single colonies and subsequent agarose gel electrophoresis (450-500 bp). Include positive and negative controls for discriminating the VHH specific bands.

9. Alternatively, the bacterial culture can be incubated overnight. In this case, phages can be produced on the next day along with glycerol stocks. To produce phages inoculate 200 ml of 2-YT with glucose and ampicillin with 4 ml of the overnight culture, enough to cover a library with the size of 10^8 CFU, and incubate at 37°C with shaking. The starting $OD_{600\,nm}$ must be 0.01-0.05. When $OD_{600\,nm}$ reaches 0.5-0.6, follow the protocol from Subheading 3.6, step 9.
10. The time varies depending on a library size (*see* Note 6). For 10^7-10^8 CFU libraries, it takes approximately 3 h. To make the outgrowth of libraries faster, 2-YT medium should be prewarmed at 37°C in advance.
11. To prepare glycerol stocks, supply the rest 200 ml of the initial culture with 100 ml of fresh 2-YT with glucose and ampicillin. Incubate for 9 h at 37°C with shaking.
12. The pellet must be slightly visible what indicates very clean solution of phages. Otherwise, additional washing step should be applied, as follows: the 1 ml of supernatant should be supplied with 250 µl 20% PEG, incubated on ice for 10 min, and centrifuged at max speed for 3 min. The pellet with phages should be resuspended in 1ml PBS and centrifuged again to remove residual bacterial cell debris. The supernatant contains purified phages.
13. Evaluation of the optimal coating concentration by ELISA with the titration of VLPs can be performed. One should consider that the concentration giving the highest signal in ELISA might not reflect the real saturation of the coating surface. Using even higher coating concentration for phage selection is strongly recommended. Always include null VLPs in selection step as negative control with the same concentration as target VLPs.
14. The overnight TG1 culture can be stored at 4°C for up to one week and used for several selection rounds.
15. After the step 17 in Subheading 3.6, the concentration of phages in the stock solution is normally 10^{13} phages/ml. For the first round of selection, the phages need to be diluted 1:10 in the blocking solution (input phages). For all the subsequent selection rounds use 1:100 dilution of phages. Prepare the phage dilutions in excess to have some leftovers for titration of the input. The high level of nonspecific background, if observed, can be reduced by pre-blocking of the phage input with additional incubation in blocking solution for 30 min at room temperature with shaking or head-over-head rotation before transferring to the selection plate.
16. If TG1 culture are not used immediately at $OD_{600\,nm}$ = 0.5, place the cells at 4°C or on ice to keep them in logarithmic phase.

Since a contamination of TG1 cells can easily occur, only use 5- or 10-ml disposable sterile stripetts to take the cells out of the culture flask. To control the contamination, grow in parallel another culture in 2-TY with addition of ampicillin. The lack of bacterial growth in this flask will evidence the lack of contamination in the night culture.

17. The low concentration of Tween-20 is used to wash VLPs (see Subheading 3.7, step 10 and 11) in more mild condition.

18. Phages can be eluted by different methods including elution with low pH or competitive elution with a high concentration of a ligand to the target protein if possible. Sometimes the aforementioned elution conditions are not harsh enough to elute strong binders and elution with trypsin is preferable. Incubation with trypsin for a short period of time (up to 1 hour at room temperature) do not affect phage infectivity